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TITLE: GUIDANCE PROTOCOL: APPLICATION OF NUCLEIC ACID-BASED TOOLS FOR MONITORING MONITORED NATURAL ATTENUATION (MNA), BIOSTIMULATION AND BIOAUGMENTATION AT CHLORINATED SOLVENT SITES

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GUIDANCE PROTOCOL

Environmental Restoration Project ER-0518

Application of Nucleic Acid-Based Tools for Monitoring Monitored Natural Attenuation (MNA), Biostimulation, and Bioaugmentation at Chlorinated Solvent Sites

January 2011

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EXECUTIVE SUMMARY

Reductive dechlorination is a promising process for biodegradation of chlorinated solvents. The successful field evaluation and implementation of the reductive dechlorination process is dependent on a comprehensive understanding of contaminant, geochemical, and microbial data. Nucleic acid-based tools are commercially available to identify relevant *Dehalococcoides* (*Dhc*) bacteria. These tools detect and quantify *Dhc* 16S rRNA genes and three *Dhc* reductive dehalogenase (RDase) genes involved in the reductive dechlorination of chlorinated ethenes. These tools were demonstrated and validated in ESTCP Project ER-0518 (Application of Nucleic Acid-Based Tools for Monitoring Monitored Natural Attenuation (MNA), Biostimulation, and Bioaugmentation at Chlorinated Solvent Sites). The application of nucleic acid-based molecular biological tools (MBTs) can result in significant cost reductions and reduced project time-lines, as users can use MBT site data to assess:

- Where long-term Monitored Natural Attenuation (MNA) will be effective;
- Where biostimulation will achieve complete dechlorination without DCE/VC "stall"; and/or
- Where bioaugmentation will be required.

For MNA sites, MBT analysis should be considered after the primary line of evidence is obtained (e.g., the groundwater plume appears to be stable or decreasing in concentrations over time) and adequate reducing conditions have been observed. It has been demonstrated that *Dhc* cell titers of 10^4/L – 10^5/L support MNA at chlorinated ethene sites.

For sites where enhanced anaerobic bioremediation (e.g., biostimulation with or without bioaugmentation) has been proposed, MBT analysis should be conducted as a part of a pre-design remedial investigation to assess the site's geochemical conditions, determine if implementation of the microbial reductive dechlorination process is a viable remedial alternative, and evaluate the possible need for bioaugmentation. *Dhc* titers below 10^4/L or a need to reduce remediation time frames indicate bioaugmentation may be needed.

There are several sampling methodologies available to field practitioners. The selection of groundwater sampling methods can significantly influence the quantification of *Dhc* biomarker genes (SERDP and ESTCP, 2005; Ritalahti et al., 2010). Sampling options should consider site-specific data quality objectives (DQOs) to determine the most appropriate method for a given

site. Ongoing SERDP-funded research will help to identify improved management practices with respect to laboratory methods for MBT analysis and will evaluate procedures to optimize collection of representative solid and groundwater samples from an aquifer.

The sampling and handling procedures described herein have been validated for *Dhc* assessment at chlorinated solvent sites. These techniques can also be applied to sites impacted with other contaminants, such as petroleum hydrocarbons or metals, for collecting microbial biomass to extract biomarkers for MBT analysis.

For the purpose of this protocol, groundwater sampling should be conducted using low-flow purging methods. To correct for sampling biases, the number of *Dhc* gene copies can also be normalized to the total number of bacterial 16S rRNA genes quantified in the same sample and reported as %*Dhc*. The %*Dhc* values can range from low fractions of percentages in samples that have low numbers of *Dhc* and high numbers of other bacteria to greater than 50% in enriched dechlorinating consortia (SiREM, 2005). Normalization of *Dhc* cell counts is most useful when evaluating temporal variation of the *Dhc* population during bioremediation performance monitoring.

Based upon field trials conducted in ER-0518 (Ritalahti et al., 2010) and guidance of commercial vendors, on-site (i.e., in the field) groundwater filtration is recommended. Field filtration increases the likelihood of collecting suspended particles, decreases shipping costs, reduces time-consuming and costly laboratory biomass collection procedures, and avoids cost for disposal of contaminated groundwater. A protocol providing a step-by-step approach to groundwater sampling during bioremediation monitoring (Ritalahti et al. 2009; Petrovskis et al. 2011) is provided and summarized in Figure 4. Methods may vary according to site-specific conditions; however, a crucial consideration is that the sampling protocol for a given well (or site) is defined and maintained for the duration of the monitoring efforts.

A *Dhc* cell titer exceeding 10^6 cells per liter is a good predictor of ethene production and complete detoxification (Ritalahti et. al, 2010; Lu et al. 2006). Due to variability in laboratory analyses, this protocol establishes a *Dhc* cell titer of 10^6 to 10^7 per liter as a target threshold for ethene production. The following guidance is provided for groundwater samples:

***Dehalococcoides* 16S rRNA gene copies per L**	**Interpretation**
$<10^4$	Low *Dhc*, efficient dechlorination and ethene production unlikely
$10^4 – 10^6$	Moderate *Dhc*, which may or may not be associated with observable dechlorination and ethene formation
$>10^6$	High *Dhc*, which is often associated with high rates of dechlorination and ethene production

Groundwater samples for MBT analysis should be collected from representative monitoring wells where geochemistry and VOC parameters are collected, although the number of samples collected for MBTs is typically lower than those selected for comprehensive geochemical and contaminant analysis. At sites slated for enhanced treatment, samples should be collected and analyzed immediately prior to injection of stimulants (i.e., biostimulation) and dechlorinating biomass (i.e., bioaugmentation).

For supporting MNA, groundwater sample locations should generally be chosen where biodegradation products have been observed and where total VOC concentrations are at least 0.1 mg/L. The sampling frequency for MNA sites should allow for collection of adequate data to support the MNA approach, but the time between sampling events for MNA is generally longer than at sites where more active (i.e., enhanced) bioremediation remedies are employed. Semi-annual or annual monitoring is recommended for MNA sites.

During performance monitoring at enhanced bioremediation sites, sampling locations should be selected to evaluate i) the distribution of amendments, ii) reductions in parent compound concentrations and production of dechlorination daughter products and ethene, iii) contaminant mobility, iv) changes in geochemistry, v) *Dhc* biomarkers abundance, and v) other factors which relate to the ongoing effectiveness of the treatment (e.g., pH). Immediately following bioaugmentation injections, MBT sampling should be conducted more frequently (e.g., monthly or quarterly) to monitor the distribution and proliferation of dechlorinating microorganisms in the treatment area. For most sites, two years of quarterly monitoring are recommended during enhanced bioremediation implementation. Post-treatment monitoring data are evaluated to determine whether system design or sampling plan revisions are necessary.

By clearly understanding how site geochemistry and *Dhc* abundance affect contaminant transformation and detoxification, MNA, biostimulation, and bioaugmentation, remedies can be designed and modified to optimize the efficiency of bioremediation treatments.

1. PURPOSE

The purpose of this document is to provide guidance to Remedial Project Managers (RPMs) and field practitioners on the application of Molecular Biological Tools (MBTs), specifically nucleic-acid based tools, for evaluating monitored natural attenuation (MNA), biostimulation and bioaugmentation at chlorinated solvent sites. This protocol summarizes the current state of the practice of these tools and is intended to provide a technically sound and practical approach to MBT use. This guidance document will be updated, based on the findings from SERDP project ER-1561 (Standardized Procedures For Use Of Nucleic Acid-Based Tools). This guidance document provides recommendations regarding sampling approaches and criteria in evaluation of data for use in bioremediation decision-making.

2. INTRODUCTION

Microbial degradation plays a primary role in the fate and transport, and ultimately the remediation of chlorinated solvents. Knowledge of the responsible microbial processes is crucial to understanding the impact of biodegradation on cleanup times at a given site. Understanding these microbial processes can assist site owners in making informed decisions to better assess contaminated sites and manage bioremediation efforts.

MBTs measure target biomarkers (e.g., specific nucleic acid or ribonucleic acid sequences, contaminant-specific isotopes, peptides, proteins or lipids) that are indicators of potential biological degradation of target contaminants. Research and development needs regarding the use of MBTs for environmental remediation were addressed in a SERDP and ESTCP Expert Panel Workshop (2005). This workshop presented a comprehensive summary of the MBT techniques, applications, issues, questions, and associated research needs. Among all MBTs, application of nucleic acid-based tools is the most advanced for application to environmental samples, and assays to detect the presence and enumerate the abundance of key dechlorinators (e.g., *Dehalococcoides* (*Dhc*) bacteria) have been designed and are commercially available. Current research efforts aim at identifying a wider range of biomarker genes to describe the dechlorinating bacterial community with greater accuracy. Recent laboratory studies suggested that peptide, protein and lipid biomarkers are also useful for monitoring target dechlorinators;

however, these tools have to mature before they can complement nucleic acid-based tools for site assessment and bioremediation monitoring. Hence, the current focus is on nucleic acid-based tools.

The integrated application and analysis of MBT, geochemical, and contaminant data is useful to determine if a biological remedy is suitable for a particular site or if a physical-chemical treatment option should be considered. Further, the application of nucleic acid-based tools allows site owners and managers to decide and focus on the most effective bioremediation strategy (i.e., monitored natural attenuation, biostimulation and/or bioaugmentation). Following technology implementation, MBTs monitor the bioremediation process and yield information to allow site management decisions for achieving cleanup goals in the most cost-effective manner and desired time frames.

3. BACKGROUND

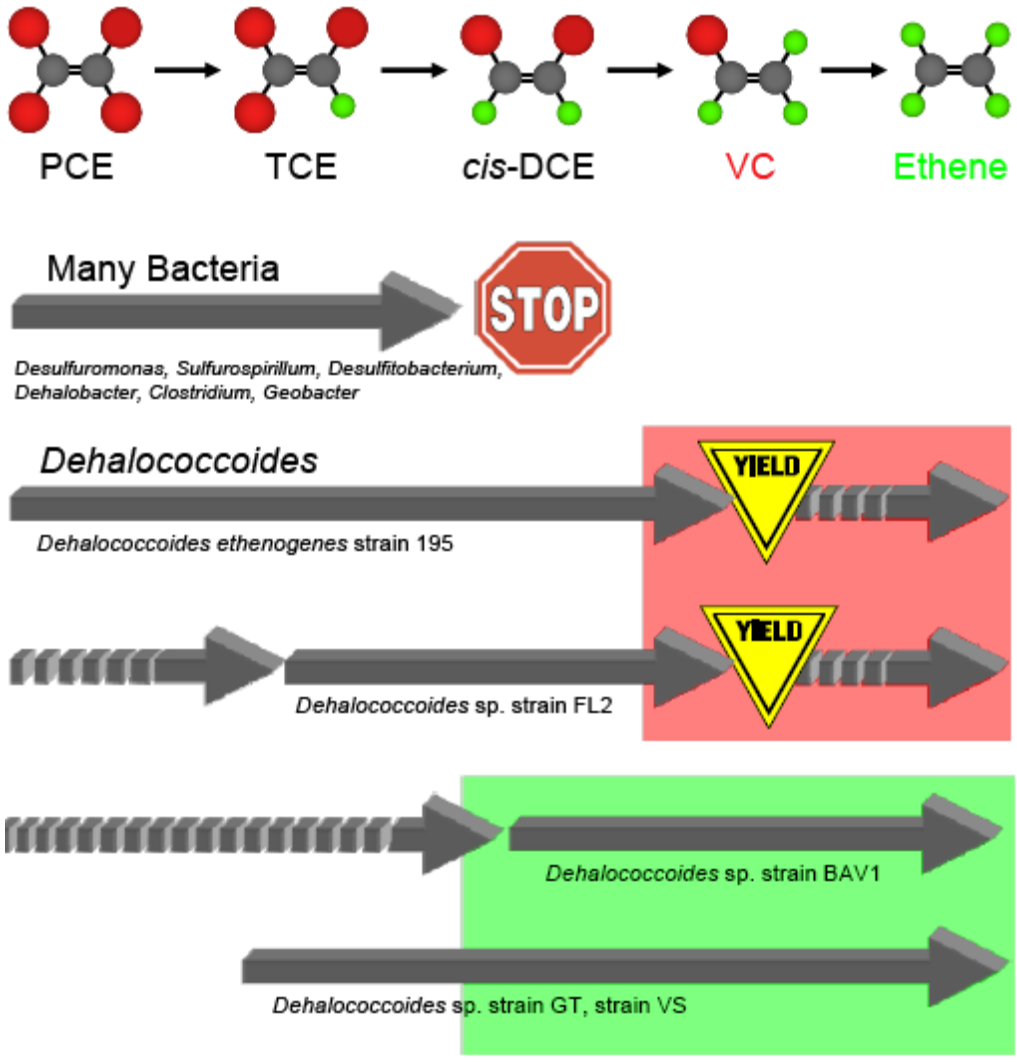
The chlorinated solvents tetrachloroethene (PCE), trichloroethene (TCE), and their transformation products *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-DCE, 1,1-DCE and vinyl chloride (VC) are common groundwater pollutants. PCE and TCE are resistant to degradation under aerobic conditions but can be reductively dechlorinated to less chlorinated ethenes and ethene under anaerobic conditions. While phylogenetically diverse bacteria dechlorinate PCE and TCE to *cis*-DCE, only members of the *Dhc* group have been demonstrated to carry out the final dechlorination steps from DCE to ethene. However, the complete dechlorination of PCE to ethene is a multi-step process and is most effectively carried out by more than one population. These specialized bacteria use the chlorinated ethenes as electron acceptors and gain energy for growth from the reductive dechlorination reactions. This process has been called (de)chlororespiration but the term organohalide respiration should be used. Figure 1 summarizes the current knowledge of microbial populations involved in the reductive dechlorination of PCE to ethene. An electron donor is required to support organohalide respiration because each reductive dechlorination step requires 2 electron and 2 protons. The chlorine substituent is released as HCl, which dissociates and forms chloride and a proton. These are relevant observations because the availability of suitable electron donors often limits the activity of dechlorinators including *Dhc*, in-situ and the formation of HCl can decrease the groundwater pH

and limit dechlorination activity. *Dhc* are active at circumneutral pH and reductive dechlorination rates decrease at lower pH with no activity below pH 5.5.

Under methanogenic conditions, ethene is sometimes reduced to ethane. VC, ethene and ethane can be mineralized to carbon dioxide under both micro-aerophilic and aerobic conditions. Generally, the anaerobic reductive pathway is the most important degradation pathway for chlorinated ethenes in field applications, and hence, is the focus of this protocol.

Figure 1: Populations Involved in Reductive Dechlorination of PCE to Ethene.

Each dechlorination step consumes two electrons and a suitable electron donor is required. Several different bacterial strains belonging to the genera *Sulfurospirillum, Desulfitobacterium, Dehalobacter*, and *Geobacter* dechlorinate PCE to *cis*-DCE as end product. Bacteria that dechlorinate PCE to *cis*-DCE are metabolically versatile and use a variety of growth substrates with the exception of *Dehalobacter* spp., which can only grow with a chlorinated compound as electron acceptor and hydrogen as electron donor. The only bacteria known to dechlorinate DCEs to VC and ethene belong to the *Dehalococcoides* (*Dhc*) group. *Dhc* strains share highly similar 16S rRNA gene sequences (i.e., are phylogenetically closely related) but exhibit distinct reductive dechlorination abilities. For example, *Dhc* sp. strain 195 and strain FL2 cannot grow with VC and VC-to ethene-dechlorination is slow and often incomplete (red box). More desirable for efficient bioremediation are bacteria such as *Dhc* strain GT and VS, which use VC as a growth substrate (green box).



A few members of the genus *Desulfitobacterium* dechlorinate PCE to TCE only; however, reductive dechlorination of PCE to *cis*-DCE is more commonly observed. Bacteria that grow via PCE to *cis*-DCE reductive dechlorination belong to the genera *Desulfuromonas*, *Dehalobacter*, *Sulfurospirillum*, and *Geobacter*. PCE-to-*cis*-DCE-dechlorinating bacteria are not rare in subsurface environments and aquifers and *cis*-DCE plumes likely reflect the activity of such microorganisms.

Dehalococcoides ethenogenes strain 195 was the first isolate shown to carry out the complete reductive dechlorination sequence leading to ethene formation (Maymó-Gatell et. al. 1997); however, the final dechlorination step, the reduction of VC to ethene, is cometabolic and slow, and does not support growth of strain 195. Another isolate, *Dhc* sp. strain FL2, also produced ethene from PCE though the PCE-to-TCE and VC-to-ethene steps were cometabolic (He et al. 2005). The first *Dhc* isolate capable of growth with VC as electron acceptor was isolated from the chloroethene-contaminated Bachman aquifer (He et al. 2003). This Bachman isolate was designated *Dhc* sp. strain BAV1 and grew with all DCE isomers and VC as electron acceptors, thus efficiently detoxifying these compounds to ethene. Strain BAV1 also dechlorinated PCE and TCE but only in the presence of a DCE isomer or VC. Unlike BAV1, isolate *Dhc* sp. strain GT and strain VS are capable of capturing energy for growth from TCE dechlorination to ethene (Müller et al. 2004; Sung et al. 2006).

To date, no other bacteria that reductively dechlorinate DCEs and VC to environmentally benign ethene have been identified. Of course, it is possible that other bacteria involved in chlorinated solvent biodegradation and detoxification will be identified; however, with the information currently available, a primary focus on the *Dhc* group for the majority of chlorinated ethene bioremediation sites is justified (Löffler and Edwards 2006, AFCEE, et al. 2004). *Dhc* bacteria play relevant roles in the attenuation of chloroorganic contaminants and details about *Dhc* biology and application for chlorinated solvent bioremediation are available in Volume 4 of the SERDP and ESTCP Remediation Technology Monograph Series (Petrovskis, et al. 2011).

The attenuation of chlorinated ethenes is dependent on several site characteristics, including hydrogeologic, geochemical, and microbiological parameters. Collecting this information and

evaluating it in a weight of evidence approach is essential in determining the applicability and performance of MNA, biostimulation, and bioaugmentation. The following references provide site assessment criteria or guidelines for evaluating site characteristics favorable for the microbial reductive dechlorination process and the detoxification of chlorinated ethenes in anoxic subsurface environments.

- Implementation of MNA (Wiedemeier 1998),
- Application of a suite of electron donors (RABITT protocol by Morse et al. 1998),
- The use of soluble carbohydrates to enhance reductive dechlorination (Suthersan et al. 2002),
- The use of emulsified vegetable oils to enhance reductive dechlorination (AFCEE, 2004), and
- Enhanced anaerobic bioremediation (AFCEE, 2004).

This guidance protocol addresses the use of MBTs to quantitatively assess the *Dhc* population at chlorinated ethene sites and aims at providing guidance to evaluate MBT data. The focus will be on *Dhc*-targeted nucleic acid-based tools that have been validated and are offered commercially. The MBT of choice for specific detection and quantification of a target DNA sequence in environmental matrices is the quantitative real-time polymerase chain reaction or short qPCR. The qPCR tool provides quantitative information about *Dhc* cells in groundwater and can identify *Dhc* cells that efficiently dechlorinate VC to ethene (Figure 1).

4. MOLECULAR BIOLOGICAL TOOLS

Used in conjunction with contaminant and geochemical data, nucleic acid-based MBTs can be utilized to develop an understanding of the potential for biotransformation and detoxification at chlorinated ethene sites. These tools assist in identifying the potential for anaerobic biotransformation at a given site, establish cause-and-effect relationships after technology implementation, and inform RPMs of the most efficient site management decisions following technology implementation. Several MBTs have been developed and evaluated under the auspices of SERDP and ESTCP. qPCR assays have been most widely used to detect and quantify genes of key bacteria. Target genes include the *Dhc* 16S rRNA gene and the functional genes encoding for reductive dehalogenases responsible for individual reductive dechlorination

reactions. Other techniques [e.g., fluorescent *in-situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and others], and microarray approaches have been developed (Table 1). These MBTs can address specific questions about target organisms and the microbial community but qPCR is the method of choice for detecting and enumerating target genes in environmental samples. The presence and abundance of target genes does not directly inform of the cells metabolic status (i.e., inactive *Dhc* cells also harbor the target genes but do not contribute to dechlorination). The quantitative assessment of RDase gene expression was suggested as a means of measuring *Dhc* activity. The rationale is that mRNA (a measure of gene activity) is only produced in cells that are actively dechlorinating. Unfortunately, mRNA quantity is a poor measure of *Dhc* dechlorination activity because non-dechlorinating *Dhc* cells maintain RDase gene transcripts (i.e., the presence and abundance of RDase mRNA are not linked with dechlorination activity) (Amos et al. 2008, Fletcher et al. 2010). Although DNA-based tools cannot provide direct evidence for activity, temporal measurements of *Dhc* target gene abundance can serve as an indirect activity measure because *Dhc* can only increase in numbers when performing reductive dechlorination reactions.

4.1 qPCR ANALYSIS FOR DECHLORINATING *Dhc* BACTERIA

To identify bacteria associated with the detoxification pathway of interest, deoxyribonucleic acid (DNA) can be extracted from site aquifer material or groundwater, and target (biomarker) genes can be specifically detected and quantified using qPCR assays.

This analysis assists in determining the potential for reductive dechlorination and predict the end product(s) of the process. PCR analyses are commercially available for approximately $300 per sample. Further advances in multiplex, high-throughput PCR analysis should reduce the cost. PCR-based approaches for detecting several PCE-to-*cis*-DCE-dechlorinating bacteria (e.g., *Desulfitobacterium, Dehalobacter, Sulfurospirillum,* and *Desulfuromonas*) are also commercially available (Löffler et. al., 2000; Hendrickson 2002; Ritalahti and Löffler 2004; Sung et al., 2006; Ritalahti et al. 2006).

A correlation exists between complete reductive dechlorination to ethene and the presence of *Dhc* bacteria (Löffler and Ritalahti, 2001; Ritalahti et al., 2002; Hendrickson et al., 2002). Unfortunately, one cannot rely solely on the detection of *Dhc* bacteria, because members of this

group have different RDase genes and therefore dechlorinating activities despite sharing highly similar or identical 16S rRNA gene sequences (He et al., 2003, Duhamel et al. 2004, Sung et al. 2006). Therefore, *Dhc* 16S rRNA gene-targeted qPCR quantifies the total *Dhc* population but cannot distinguish between *Dhc* strains with different dechlorination activities (i.e., *Dhc* strains that efficiently reduce VC versus *Dhc* strain that cannot grow with VC as electron acceptor). To overcome the limitations of the 16S rRNA gene approach, functional genes coding for reductive dehalogenases (RDases) involved in specific dechlorination step(s) must be identified for accurately monitoring the process of interest. Four RDase genes involved in chloroethene dechlorination have been identified in *Dhc* bacteria: *pceA* encoding a PCE-to-TCE RDase and *tceA* coding for a TCE RDase, both present in strain 195 (Magnusson et al. 2000); *tceA* in strain FL2 (He et al., 2005), *vcrA* coding for a VC RDase present in strain VS (Müller et al., 2004) and strain GT (Sung et al., 2006), and *bvcA* coding for another VC RDase in strain BAV1 (Krajmalnik-Brown et al., 2004). Analysis of these functional genes provides more direct information about the key dechlorinators; however, additional RDase genes involved in chloroethene dechlorination exist, and efforts are underway to identify additional *Dhc* RDase genes to more comprehensively assess and monitor reductive dechlorination processes.

qPCR allows for sensitive detection and quantitative analyses of biomarker gene copies of dechlorinating bacteria of interest (i.e., *Dhc*). As this tool is now commercially available and has been applied for bioremediation monitoring for years, correlations have been developed for threshold numbers of *Dhc* biomarker gene copies and dechlorinating activity. Note that other qPCR assays can be adopted to quantify messenger RNA (mRNA) as a measure of gene expression, and therefore activity. The measurement of mRNA of *Dhc* RDase genes would inform of RDase gene expression and hence, only measure active *Dhc* cells, and, at least theoretically, the mRNA abundance should correlate with dechlorination activity (i.e., rates). Although promising, recent findings suggest that RDase mRNA abundance in *Dhc* cells correlates poorly with dechlorination activity.

This protocol will focus on the currently available *Dhc* biomarker genes (i.e., DNA) and qPCR. More useful biomarker genes to monitor reductive dechlorination processes await discovery and multiple research groups address this research need. Nevertheless, the qPCR analysis of the *Dhc* 16S rRNA gene and the *tceA*, *vcrA* and *tceA* gene provide useful information of the *Dhc*

population responsible for the reductive dechlorination of chlorinated ethenes, and the existing qPCR protocols can easily be expanded to include the analysis of additional biomarker genes.

5. APPLICATION OF qPCR IN BIOREMEDIATION EVALUATION

The following two sections provide a general protocol for the use of qPCR for evaluation of MNA and biostimulation/bioaugmentation.

Guidance for interpreting *Dhc* test results has been provided by commercial vendors. qPCR analysis of the *Dhc* 16S rRNA gene is generally interpreted as the number of cells present in the sample. This is justified because all known *Dhc* cells possess a single 16S rRNA gene, and the number of *Dhc* 16S rRNA genes quantified equals the number of *Dhc* cells. Similarly, the known biomarker RDase genes occur as single copy gene on the known *Dhc* genomes. The following guidance is provided for groundwater samples:

***Dehalococcoides* 16S rRNA gene copies per L**	**Interpretation**
$<10^4$	Low *Dhc*, efficient dechlorination and ethene production unlikely
$10^4 – 10^6$	Moderate *Dhc*, which may or may not be associated with observable dechlorination and ethene formation
$>10^6$	High *Dhc*, which is often associated with high rates of dechlorination and ethene production

Through ESTCP project ER-0518 (Ritalahti et al. 2010) and other research (Lu et al. 2006), *Dhc* 16S rRNA or *vcr*A gene copies of 10^6 to 10^7 per liter of groundwater have been found to strongly correlate with complete detoxification and ethene production.

It should be noted that complete detoxification (i.e., removal of all chlorinated ethenes) can be observed without the production of ethene. For one thing, ethene is less tractable with the contemporary analytical procedures than the chlorinated ethenes, and the focus is on contaminant removal rather than ethene production. Further, alternative processes, such as anaerobic VC or ethene oxidation (Bradley and Chapelle, 2000), or aerobic oxidation at low oxygen thresholds

(Gossett, 2010), can contribute to VC and ethene removal. Therefore, field data will rarely generate closed mass balances.

The quantification of *Dhc* is highly dependent on the sampling method (SERDP and ESTCP, 2005). For the purpose of this protocol, groundwater sampling should be conducted using low-flow purging methods (Ritalahti, 2009). To correct for sampling biases, the number of *Dhc* gene copies can also be normalized to the total number of bacterial 16S rRNA genes and reported as %*Dhc* (Ritalahti et al. 2006). The %*Dhc* values can range from low fractions of percentages in samples with low *Dhc* to greater than 10% in groundwater form field sites undergoing bioremediation. Normalization of *Dhc* cell counts is most useful at a site when evaluating temporal variation of *Dhc* during bioremediation performance monitoring.

5.1 qPCR USE IN SUPPORTING MONITORED NATURAL ATTENUATION

Monitored natural attenuation (MNA) is a technically feasible and cost-effective remedial action for many chlorinated solvent groundwater plumes. It is recommended that at least the following three lines of evidence (Wiedemeier et al. 1998) are used to support MNA:

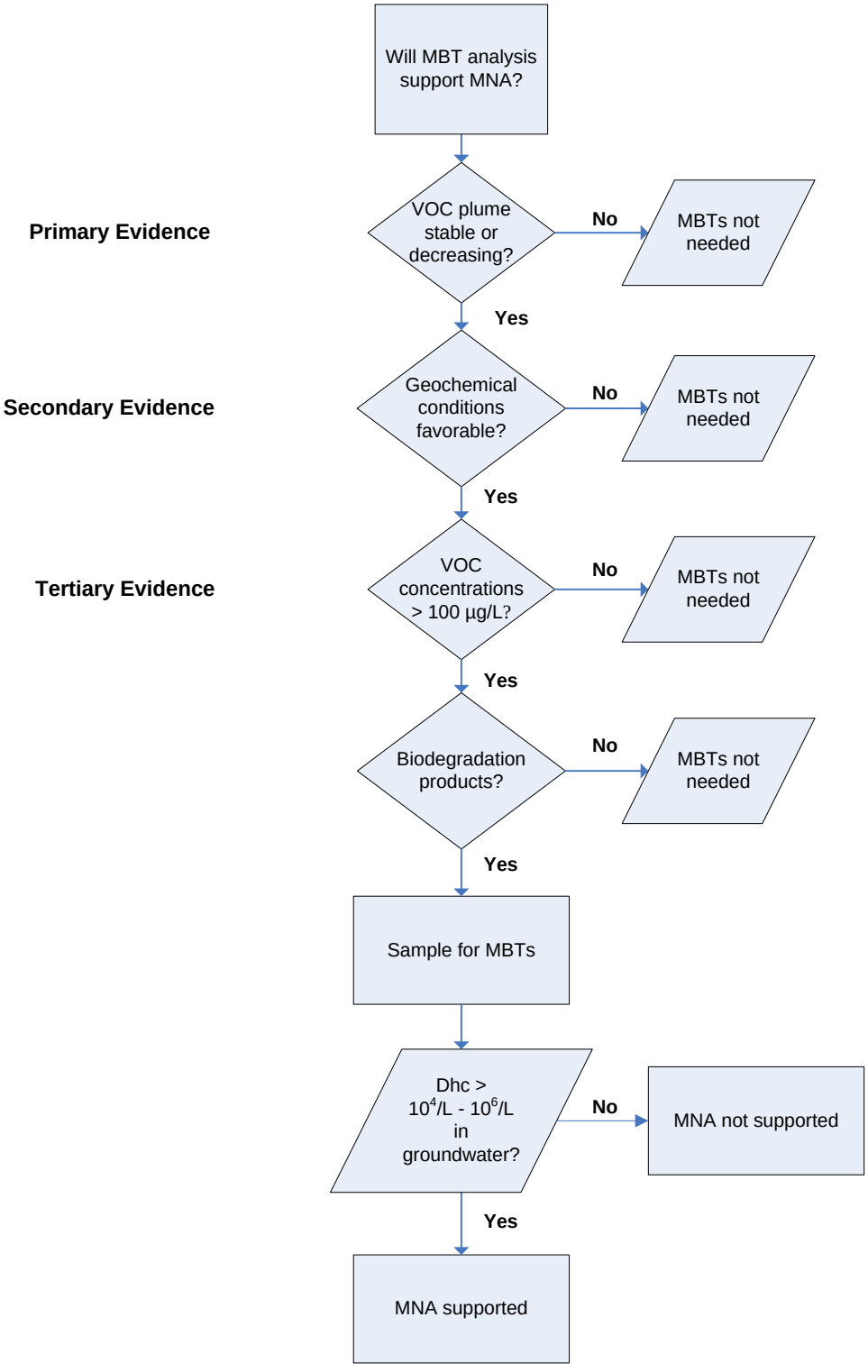
- Primary: stable or decreasing groundwater VOC plume concentrations;
- Secondary: favorable geochemical conditions (i.e., iron reducing, sulfate reducing, and/or methanogenic conditions); and,
- Tertiary: microbiological evidence supporting biodegradation.

qPCR provides the most rapid method for providing evidence that microbes capable of reductive dechlorination of chlorinated ethenes are present. As the use and limitations of qPCR become better understood, the importance of this data will increase, potentially replacing surrogate geochemical measures of activity. Figure 2 provides guidance to site owners/managers on whether to apply/not apply qPCR as part of their remediation strategy. The protocol assumes that primary lines of evidence (a stable plume and favorable biogeochemical conditions) suggest that the reductive dechlorination process is feasible at the site.

For supporting MNA, groundwater sample locations should be chosen where biodegradation products have been observed and where total VOC concentrations are at least 100 µg/L. Higher

concentrations are typically observed near the source area, with lower concentrations in the associated plume. At lower VOC concentrations (less than 100 µg/L), such as in the downgradient plume areas, organohalide-respiring bacteria may not be present in high numbers due to low electron acceptor (chlorinated ethenes) concentrations, electron donor limitations, or unfavorable geochemical (e.g., oxic) conditions. The flow chart presented in Figure 2 presents a general framework to help RPMs to decide on the value of MBT application at MNA sites. Of course, MBT application may provide useful information even if the flow chart recommends "MBTs not needed" in cases where alternative microbial remedies (e.g., biostimulation) are being considered. Under such scenarios, MBT analysis may be very useful to decide on the most promising enhanced biological remedy.

Figure 2: MBT Analysis in Support of MNA at Chlorinated Ethene Sites



5.2 qPCR USE IN SUPPORTING BIOSTIMULATION AND BIOAUGMENTATION

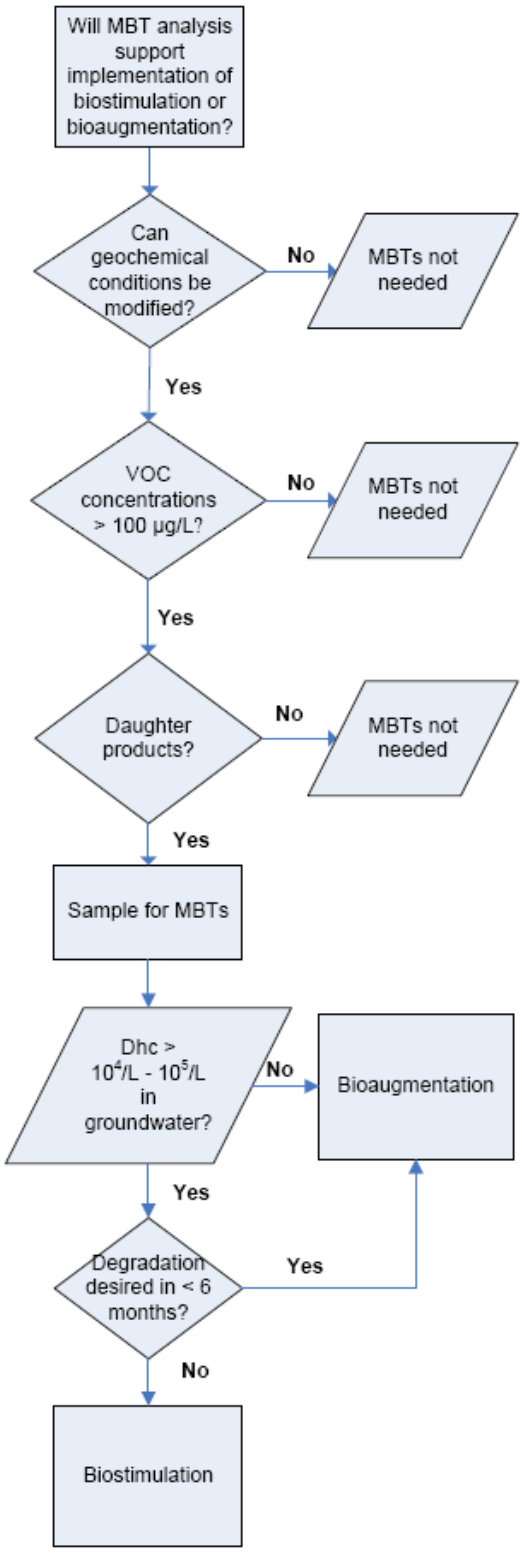
Enhanced anaerobic bioremediation has been implemented with varying degrees of success (AFCEE, 2004). In some cases, an accumulation of intermediate daughter products, such as *cis*-DCE and VC, may occur due to insufficient amount of electron donor, inappropriate geochemical conditions (e.g., aerobic, low pH), and/or the lack of sufficient *Dhc* bacteria responsible for complete reductive dechlorination to ethene. Biostimulation coupled with bioaugmentation has been implemented to address incomplete transformation and accumulation of toxic intermediates (i.e., daughter products).

qPCR assays to quantify *Dhc* cells during bioremediation are mature, commercially available, and their value for decision-making has been repeatedly demonstrated. For example, soil and groundwater samples were collected from different locations inside a pilot test plot at the Bachman Road chlorinated ethene site in Oscoda, Michigan. The data indicated that *Dhc* bacteria were present in high numbers at locations where complete dechlorination to ethene occurred. On the other hand, *Dhc* bacteria were present in low numbers, or were not detectable, in locations where groundwater measurements indicated activity *cis*-DCE/VC stall. qPCR application showed that the *Dhc* population size increased following biostimulation, demonstrating engineered *Dhc* growth under field conditions (Lendvay et al. 2003).

MBTs can also be used during feasibility studies to evaluate remediation technologies and design pilot tests. For example, at a TCE-contaminated site, qPCR analysis and microcosm studies indicated that bioaugmentation would be required for successful remediation. This was confirmed during lactate injection in the field pilot test, where *cis*-DCE accumulation was observed until the site was bioaugmented. Following bioaugmentation, TCE and *cis*-DCE were dechlorinated to VC, which was removed to non-detectable concentrations with negligible ethene production. qPCR data demonstrated that the injected *Dhc* strains increased *in situ* following bioaugmentation and *Dhc* cell titers correlated with reductive dechlorination activity (Seguiti et al. 2006).

Figure 3 presents a flowchart for using qPCR in design of enhanced bioremediation systems. If geochemical conditions are favorable (i.e., iron reducing, sulfate reducing, and/or methanogenic), VOC concentrations are greater than 100 μg/L, and dechlorination products are observed, then qPCR analysis can be used to assist in determining the necessity of including biostimulation with or without bioaugmentation for effective site bioremediation. The decision to bioaugment depends on several factors, such as desired remediation time frames, the ability to inject cell suspensions into the subsurface, and the hydraulic characteristics of the contaminated subsurface (AFCEE, 2004).

Figure 3. MBT Analysis in Support of Biostimulation/Bioaugmentation at Chlorinated Ethene-Contaminated Sites



6. FIELD SAMPLING METHODS

DNA may be extracted from soil/aquifer material or groundwater samples for the quantification of relevant dechlorinating (i.e., organohalide-respiring) bacteria (i.e., *Dhc*). Due to the spatial variability of microbes in aquifers and inadequate sampling approaches to address this variability, false negative results are possible, and a sufficient number of samples must be analyzed to build confidence in the analytical results. The selected groundwater sampling method significantly influences the quantification of *Dhc* (SERDP and ESTCP, 2005). The following factors can influence microbial quantification in groundwater samples:

- Laboratory QA/QC procedures and standardization
- Well age and materials of construction
- Sampling technique and procedures
- Sampling frequency
- Groundwater and atmospheric temperature effects
- Sample turbidity
- Geochemical environment
- Chlorinated ethene concentrations in groundwater
- Sample processing and shipping

The sampling and handling procedures described herein have been validated for *Dhc* assessment at chlorinated solvent sites. These techniques can also be applied to sites impacted by petroleum hydrocarbons or metals for collecting microbial biomass for MBT application.

It is currently unclear whether the majority of *Dhc* cells and other dechlorinating bacteria are bound to soil particles or freely suspended in groundwater. Preliminary work in column studies indicates that *Dhc* can be identified primarily as planktonic cells (un-attached to surfaces) in the aqueous phase, although a fraction of the *Dhc* population occurs associated with solids (Cápiro et al. 2010). The quantification of *Dhc* may be influenced by many factors; however, normalization between samples is possible by determining total bacterial 16S rRNA genes with qPCR and expressing the *Dhc* 16S rRNA genes (i.e., *Dhc* cells) as a proportion of total bacterial 16S rRNA genes in the sample. The methods described below should be considered and selected based on site-specific objectives and the same methods should be used for samples bound for

contaminant and geochemical analyses. Selection of the most appropriate sampling method is outside the scope of this protocol. It is recommended that the appropriate Federal, state, and/or local guidance be reviewed and adhered to before selecting and implementing the most appropriate method for a given site. The same method should be used throughout the evaluation of a given site because changes in the groundwater sampling protocols over the course of the monitoring effort will render comparative data analysis difficult or impossible. Therefore, MBT data should only be compared when the same sampling methods were applied. Below is a list of possible groundwater sample collection procedures and some advantages and disadvantages of each.

High-flow Purge

Historically, the most common method of collecting groundwater samples involves using bailers or high-speed pumps to purge three to five casing volumes prior to sample collection. Although this method agitates the water column and can mobilize sediment, the sample stream is often cleared up by the time the purge is complete (Stroo et al. 2006). Therefore, this method may decrease the turbidity of the sample, which may result in lower concentrations of *Dhc* bacteria in the sample.

Low-flow Purge

Low-flow purging (100-500 mL/min) is generally recommended to collect a representative sample prior to contaminant or geochemical analysis (Puls and Barcelona, 1996). These low turbidity samples may underestimate *Dhc* cell titers in the subsurface because the methods are derived to collect low turbidity samples. This is of particular concern if VOC and geochemical data suggest that biotransformation to ethene is occurring and *Dhc* cell titers are falsely underestimated or not detected (i.e., false negative). Some commercial vendors recommend surging the well after parameter stabilization to increase the turbidity prior to collection of a groundwater sample for microbial analysis (SiREM 2005). The suspension of sediment after surging is likely derived from the well sump, which may not be representative of the flow zone of interest across the well screen.

Passive Sample Collection

Passive sample collection involves insertion of a retrievable device into a monitoring well for collection of a groundwater sample or for development of a microbial biofilm on the medium for subsequent laboratory analysis. This method may or may not represent the actual *Dhc* titer in the aquifer. One limitation of this approach is that the quantification of *Dhc* should be normalized to total bacterial biomass, limiting the interpretation of the result. *Dhc* cell titers may be affected (i.e., increased or decreased) by the matrix, which is colonized by bacteria (native sediment vs. artificial support media). Retrievable groundwater sampling devices and sophisticated biotraps are commercially available.

Sump Sediment Collection

Collection of sediment from the groundwater monitoring well sump is intended to gather fine-grained particulates that originated from the aquifer matrix. This procedure presupposes that *Dhc* exist predominantly surface-associated (i.e., attached to aquifer solids), and collection of the sump sediment resembles the colonized surface of aquifer solids. The sample procedure involves placing the intake of the sample tubing at the bottom of the well and field filtering the slurry that is pumped to land surface. This procedure overcomes some of the limitations associated with low flow sampling and multi-casing well purges that may result in lower turbidity and therefore potentially increase detection of *Dhc* cells. The *Dhc* cell numbers should be normalized to total bacteria rather than a volume of water filtered. Potential drawbacks associated with this procedure include the contaminant flow zone of interest may not intersect with the bottom of the well and/or the influence of diffusion of stagnant water in the monitoring well riser into the well sump that does not reflect the geochemical conditions in the aquifer. These two conditions may affect the MBT analysis and the results may not reflect the *Dhc* abundance in the aquifer.

6.1 COLLECTION OF SAMPLE VOLUME AND SAMPLE FILTRATION

As noted earlier, one limitation of groundwater sampling for bacteria is the inability to determine whether the planktonic cells in a groundwater sample are representative of the true abundance in the aquifer formation. In addition to the sampling methods for groundwater described above, the approach for biomass collection must also be considered. There are two commonly recognized methods for biomass collection from groundwater: 1) A relatively large volume (e.g., more than

1 liter) of groundwater is collected and shipped to the analytical laboratory for biomass collection and, 2) the Groundwater is filtered in the field (i.e., on-site). Each of these options has advantages and disadvantages. For example, collection of large volumes of water requires shipment of large volumes, which is costly, requires laboratory handling of large volumes of contaminated water and proper disposal. Based upon field trials conducted in ER-0518 (Ritalahti et al., 2010) and guidance from commercial vendors, field filtration is recommended. Field filtration decreases shipping costs, reduces costly laboratory extraction procedures, and avoids off-site disposal of contaminated water. Details of the on-site groundwater filtration procedure are provided in Ritalahti et al. 2009, Ritalahti et al. 2010, and Petrovskis et al. 2011.

The following protocol provides a step-by-step approach to groundwater sampling (Ritalahti et al. 2009; Petrovskis et al. 2011). The protocol is summarized in Figure 4. Methods may vary according to site-specific conditions; however, a crucial consideration is that the sampling protocol for a given well (or site) is defined and maintained for the duration of the monitoring efforts. Changes to the protocol during monitoring will complicate data interpretation and should be avoided.

- Connect a flow-through cell and hand held multiparameter instrument to a low-flow pump (e.g., peristaltic pump) and begin purging. Record the start time and field measurements for pH, oxidation-reaction potential (ORP), specific conductance, temperature, dissolved oxygen, and turbidity.
- Disconnect the flow-through cell after parameter stabilization.
- Optional if low cell titers are expected: Lower a disposable polyethylene bailer into the well to the midpoint of the screen and move the bailer up and down within the water column to surge the well. It is important to agitate at the midpoint of the well screen to avoid stirring up sediment in the sump and/or the bottom of the well. While continuing to surge the well with the bailer, re-connect the flow-through cell and record the field measurements for pH, ORP, specific conductance, temperature, dissolved oxygen, and turbidity until stabilization of geochemical parameters is achieved. Disconnect the flow-through cell but continue to surge the well with the bailer through the sample collection process.
- In order to sample groundwater for off-site biomass collection, fill the appropriate sample containers (e.g., clean, sterile 1-liter amber glass or plastic bottles with Teflon-lined caps, no preservatives added) directly from the effluent end of the pump. The bottles should be filled

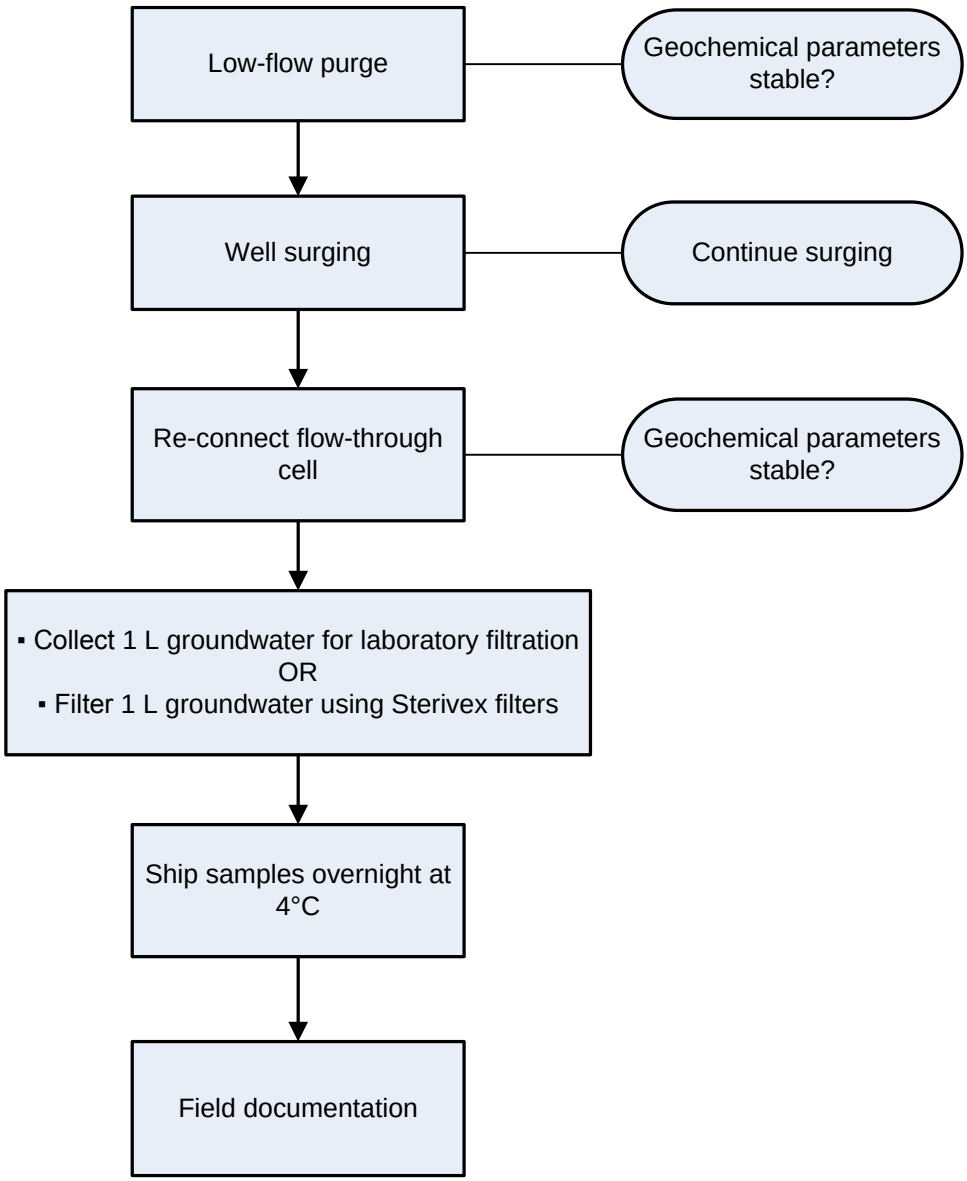
with groundwater from tubing that has already been used to withdraw one to two well volumes of groundwater to ensure that a representative sample of aquifer water, rather than well water, is collected. The bottles should be filled to capacity (i.e., minimal headspace) to minimize air exposure. Apply the Teflon-lined caps and ensure a tight seal.

- For on-site biomass collection (recommended), use sterile 0.22 µm membrane filter cartridges. Attach 1/4 to 5/16-inch polyethylene tubing to the inlet of the sterile filter cartridge and secure with a clamp. Place the cartridge over a graduated cylinder that can accurately measure the volume of water filtered. Ideally, 0.5 to 2 liters of water are collected; however, depending on groundwater characteristics, up to 10 liters are filtered, and as little as 10 mL may be sufficient for subsequent biomarker analysis. Using a 10-mL syringe filled with air, push any remaining liquid out of the sterile filter cartridge. Close the inlet and the outlet of the filter cartridge with male and female leak-proof plugs, respectively. If needed, replicate samples should be collected consecutively without flow interruption. Record the volume of filtered groundwater on the chain-of-custody form and on the filter cartridge barrel with a black permanent marker, and transfer each capped filter cartridge to a separate, new 50-mL conical plastic tube.
- Immediately after sampling, transfer samples to coolers with ice packs and/or blue ice (in plastic freezer bags) to ensure refrigeration at 4 ˚C until arrival at the analytical laboratory. Falcon tubes (50 mL) or equivalent containers are used for protecting the filter cartridges during shipping and storage. Use additional packing material, as appropriate, to prevent movement and breakage during shipping, and place each sample in separate plastic freezer bags. The coolers with samples should be shipped for next day delivery to the analytical laboratory. It is important to notify analytical laboratories when samples are shipped to avoid delays in handling and processing that could affect biomarker integrity.
- Immediately following sample collection, record the sampling well location, the well ID, notes on individual samples (e.g., the volume of water that passed through each filter cartridge), date and time of sampling, and the type of analyses requested. Standard chain-of-custody forms must accompany each sample shipment.

SERDP-funded research is ongoing to standardize laboratory procedures and to evaluate field sampling methods to ensure collection of representative samples from an aquifer. Sampling

options should be considered with site-specific data quality objectives (DQOs) in mind to determine the most appropriate method for a given site.

Figure 4. Groundwater Sampling Protocol



6.2 SAMPLING LOCATIONS AND FREQUENCY

For MNA sites, MBT analysis should be considered as a tertiary line of evidence after the primary line of evidence is obtained (e.g., the groundwater plume appears to be stable or decreasing in concentrations over time) and adequate reducing conditions have been observed. This will allow selection of sample locations most relevant for evaluating the microbiology contributing to chlorinated ethene detoxification within the plume area. The sampling frequency for MNA sites should allow for collection of adequate data to show that the lines of evidence (Figure 2) continue to support a MNA approach, but the time between sampling events for MNA is generally less than sites where more active (i.e., enhanced) bioremediation remedies are employed. Semi-annual or annual monitoring is recommended.

For sites where enhanced anaerobic bioremediation has been proposed, MBT analysis should be conducted as a part of a pre-design remedial investigation to assess the site microbiology contributing to the transformation and detoxification of chlorinated ethenes within the plume area and evaluate the potential need for bioaugmentation. During remediation performance monitoring, MBTs should be used to evaluate the effectiveness of the treatment and the need for revising system design or monitoring strategy. Immediately following bioaugmentation injections, MBT sampling should be conducted more frequently (e.g., monthly or quarterly) to monitor the distribution and proliferation of dechlorinating bacteria (i.e., *Dhc*) in the treatment area. Two years of quarterly monitoring are recommended during bioremediation implementation. Post-treatment monitoring data is evaluated to determine whether system design or sampling plan revisions are necessary.

Factors to consider for determining the appropriate number of samples, sampling locations and frequency are considered in Table 2. As always, site-specific project objectives should be taken into consideration when selecting the number, location, and sampling frequency. Generally, samples for MBT analysis should be collected from monitoring wells where the geochemistry and VOC parameters are collected, although typically not all samples are subjected to MBT analysis.

Table 1
Summary of Molecular Biological Tools and Applications

Tools	Comments	Summary/Use
Direct/Nested PCR	Easy to perform but has false negatives	Replaced by qPCR
qPCR (16SrRNA gene) (quantitative real time Polymerase chain reaction)	Determines presence/absence/ abundance	Commercially available - widespread use
qPCR mRNA (ribosomal ribonucleic acid)	Gene expression, but unstable	More R&D needed; perhaps the future of MBTs
qPCR (target genes)	Determines presence/absence/ abundance	Commercially available - widespread use
DGGE (denaturating gradient gel electrophoresis)	Community screening, quanitative, but maybe inconclusive	Specialized use, replaced by qPCR
PLFA (phospholipid fatty acid analysis)	Community screening (viable biomass and general bacterial groups); but maybe inconclusive	Used to determine biomass, screening of high level community analysis
CSIA (compound specific isotope analysis)	Distinguish biological v. non-biological degradation, good deal of potential	Not yet commercially available, very powerful tool.

Notes:
(1) SERDP and ESTCP Expert Panel. 2005. Workshop on Research and Development Needs for the Environmental Remediation Application of Molecular Biological Tools. http://docs.serdp-estcp.org/index.cfm

Table 2. MBT Sampling Locations and Frequency

Components of Sampling Plan	Factors to Consider	Explanation	Guidance
Number of Samples	Vertical and aerial extent of plume. Border of compliance. What are the goals: source zone remediation, establishment of a biobarrier, or treatment of the entire plume?	The number of MBT samples is partial dependent on the volume/size of the plume and the remedial goals (e.g., the clean up target area).	The goal of the MBT analysis must be clearly defined. The number of samples should be suffice to clearly establish cause-and-effect relationships, guide site management decisions, and to be accepted by regulatory agencies as a line of evidence for attenuation.
	Variability of data used to characterize and delineate plume.	The variability of the VOC data across the plume may be an indicator of the expected variability of the MBT sample results.	The number of MBT samples should be sufficient to document expected variability in MBT results.
Sample Locations	Plume shape and expansion in relation to source area.	Does the plume have a simple elliptical shape emanating from a single source area or does it have an irregular shape with one or more source areas?	MBT samples should be collected from locations so that the results are representative of the area targeted for remediation.
	Distribution of contamination within stratified/heterogeneous aquifers.	Is the plume contained in one homogenous aquifer or is it contained in multiple stratified aquifers separated by low permeability units.	MBT samples should be collected from each aquifer/unit containing the plume.
	Distribution of indicator parameters throughout the target area (i.e. biodegradation products, DO, ORP).	Does the distribution of indicator parameters, such as biodegradation products, oxygen, and ORP, indicate that there are distinct biodegradation zones in the plume?	MBT results must be performed in an integrated manner and include site geochemical parameters collected simultaneously. At least one sample should be collected from each distinct biodegradation zone.
Sample Frequency	Seasonal variability of groundwater data (i.e. VOCs, oxygen, ORP).	Is there seasonal variability of the existing groundwater data, such as VOC concentration, oxygen, and ORP, that indicate the potential for seasonal variability of the MBT data?	The sample frequency should be sufficient to document expected seasonal variability of MBT results.
	For active remediation systems, frequency of electron donor injection, observed biodegradation rates, location of monitoring wells relative to injection points and groundwater flow velocity, and remediation goals	For enhanced bioremediation performance monitoring, a baseline should be established prior to any treatment. Donor injection will rapidly affect the richness and eveness of the microbial community.	Sampling should be conducted more frequently (monthly or quarterly) following bioaugmentation to monitor the distribution of dechlorinators and the establishment of dechlorinating activity in the

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